MOLECULAR CHARACTERIZATION OF GROUP A ROTAVIRUSES PREVALENT IN HIMACHAL PRADESH

SYNOPSIS

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Introduction

Diarrhea is a major healthcare concern as it is responsible for a significant proportion of morbidity and mortality around the globe. According to estimates, more than one million deaths per year are attributed to diarrhea making it the second largest killer of the children across the world [1]. The major etiological agents of diarrhea include viruses, bacteria and protozoan parasites. The role of viral pathogens in diarrhea is well established and several diarrheagenic viruses have been reported till date [2, 3]. The disease burden associated with the viral gastroenteritis has been well documented in the studies from all over the world [4, 5].

Rotavirus, the RNA virus belonging to the family of *Reoviridae* is the major viral agent which is responsible for diarrhea basically in children below the age of 5 years. Globally, rotavirus is responsible for around 2 million hospitalizations [6] and approximately 0.2 million deaths annually [7]. The prodigious disease burden attributed to rotavirus can be judged by the fact that rotavirus alone is responsible for around 40% of diarrhea associated hospitalizations [8]. India is the leading nation in terms of number of rotavirus infection associated deaths and accounts for 22% of the global mortality due to rotavirus [7]. In a recent report from India, John and co-workers have shown that rotavirus was responsible for 78,583 deaths, 872,000 hospitalizations, 3,270,000 outpatient visits and 11.37 million episodes of diarrhea in children [9]. These figures reflect the substantial burden of rotavirus disease in Indian population. Although rotavirus is recognized as a pathogen responsible for diarrhea associated illness among children at large, but reports have documented its ability to cause diarrhea in adults also [10, 11]. However, the data related to rotavirus gastroenteritis in adults is limited.

Norovirus is also a major viral pathogen associated with diarrhea among all age groups in humans. These single stranded positive sense RNA viruses are the leading cause of

gastroenteritis outbreaks and are important agents involved in sporadic diarrheal cases [12, 13]. Out of the six (GI-GVI) genogroups of noroviruses, GII is accountable for majority of the norovirus associated gastroenteritis cases, followed by GI [14, 15].

Enteroviruses are single stranded RNA viruses, belonging to the *Picornaviridae* family and they are responsible for a number of chronic and acute diseases in humans [16]. Enteroviruses are in fact a neglected pathogen for diarrhea but recent reports have showed its significance as a diarrheagenic virus particularly in children [17-20]. Keeping in view the mortality, morbidity and financial burden attributed to these viruses, it is imperative to study their epidemiology. This will be crucial in formulating the preventive strategies accordingly.

Among various viruses, rotavirus is the single most important etiological agent of diarrhea and requires special attention. Rotavirus is a wheel shaped virus having a segmented genome comprising of 11 segments of double stranded RNA (Fig. 1). The genome encodes for six structural (VP1, VP2, VP3, VP4, VP6, VP7) and six non structural proteins (NSP1-NSP6). The genome is enclosed in a triple layered capsid. The inner most layer is made up of VP1, VP2 and VP3; the intermediate layer is composed of VP6 protein; and VP4 and VP7 proteins assemble to form outermost shell of the virus. Rotaviruses are classified serologically into eight groups (A-H) on the basis of VP6 reactivity [21]. Group A, B and C rotaviruses are reported to infect humans where group A rotaviruses are accountable for majority of the morbidity and mortality in humans.

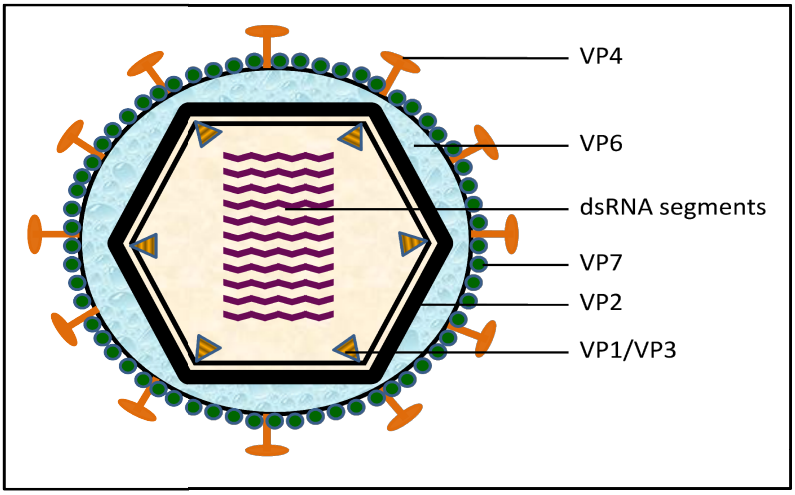


Fig. 1: Schematic representation of Rotavirus structure

The VP4 and VP7 proteins/encoding genes are used to genotype the rotaviruses in the G/P-genotyping system. The VP7 (**G**lycoprotein) protein corresponds to G-type whereas VP4 (**P**rotease sensitive protein) protein represents the P-type [22]. According to recent reports, 32 G-types (G1-G32) and 47 P-types (P[1]-P[47]) of rotaviruses have been identified [23]. The most common genotypes isolated from humans are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] [24]. However, a number of other genotypes have also been detected in significant fractions from time to time [25-27]. The relative frequency of the various rotavirus genotypes varies with time and geographical location on the globe [28]. The other factors such as vaccine pressure also leads to the changing pattern in diversity of circulating rotaviruses [29].

Rotaviruses are continuously evolving due to four major mechanisms: point mutation, interspecies transmission, reassortment events and gene rearrangement [29]. The mutations in the antigenic regions of VP7 and VP4 proteins lead to antigenic shift and drift mechanisms which give rise to antibody escape mutants. The VP7 glycoprotein contains 326 amino acids and spans across nine divergent regions (VR1-VR9) which are variable among the various genotypes [30]. Four of the divergent regions, VR5 (region A; aa 87–101), VR7 (region B; aa 141–151), VR8 (region C; aa 208–224) and VR9 (region F; aa 235–242) are considered to be the major antigen sites on the VP7 protein [31-33]. As reported by Aoki et al., two regions, 7-1 (includes epitopes from antigenic Regions A and C) and 7-2 (includes antigens from antigenic region C) are the sites of mutations that allow escape from neutralizing antibodies [34].

Currently, two rotavirus vaccines, Rotarix and RotaTeq are in use to protect from rotavirus associated complications globally. Rotarix is a live, attenuated vaccine having monovalent G1P[8] human rotavirus strain [35]. The pentavalent vaccine, RotaTeq contains human-bovine reassortant rotavirus strains and have G1, G2, G3, G4 and P[8] genotypes which are commonly found in humans [36]. The G1 component of the Rotarix vaccine belongs to the Lineage-2 whereas RotaTeq- G1 belongs to Lineage-3 [37]. Recently an Indian biotech company developed an indigenous rotavirus vaccine ‘Rotavac’ which is serially passaged attenuated human-bovine reassortant rotavirus with G9 and P[11] antigens. The phase III trials of the vaccine have shown encouraging efficacy and makes it a great candidate to be included in the Universal Immunization Programme (UIP).

India has recently introduced rotavirus vaccine in its UIP. It is very crucial for the success of a vaccination programme to have a thorough knowledge of the epidemiology and strain diversity of the targeted virus in the region. The pre-vaccination data also helps in studying the post-vaccination effect on strain diversity due to vaccine pressure. A large number of reports have documented the prevalence and distribution of circulating genotypes of rotavirus from different parts of the country. But the northern, mountainous state, Himachal Pradesh is unexplored in this context. Apart from two reports by Ramachandran et al. (1996) [38] and Jain et al. (2001) [39], there is no recent data on the prevalence and diversity of RVA strains circulating in Himachal Pradesh. Also, the samples studied from this region were very less (7 and 37). Therefore, this study aims to get an insight into the prevalence of rotavirus along with enterovirus and norovirus in this region and assess the genotypic diversity of prevalent rotavirus strains in children and adults. Also, we have carried out *in silico* analysis to study the interaction behavior of rotavirus VP7 specific antibody with the VP7 protein of the isolates and compared them with the VP7 protein of the vaccine strains.

Objectives:

Objective 1: To generate a preliminary data regarding the prevalence of Rotavirus, Norovirus and Enterovirus in diarrheic patients in Himachal Pradesh.

Objective 2: To study the epidemiological, clinical and seasonal characteristics of rotavirus infection.

Objective 3: To characterize the rotavirus strains for studying their molecular epidemiology and diversity.

Objective 4: To carry out comparative analysis of the rotavirus G1 isolates and vaccine strains for studying the effect of mutations on antigen-antibody binding using *in silico* approach.

Materials and methods

Sample collection and transportation

A total of 607 stool samples were collected from four hospitals located in Himachal Pradesh: Regional Hospital, Solan; Indira Gandhi Medical College (IGMC), Shimla; Regional Hospital, Nalagarh and Regional Hospital, Bilaspur from 2012-2015. Out of these samples, 247 were collected from the children (≤5 years), 50 were from older children (6-17 years) and 310 were from adult (≥18 years) patients. The hospitalized patients included in the study had diarrhea (defined as passage of three or more loose stools per day) as the common symptom along with vomiting, dehydration, fever and abdominal pain. The demographic details of the patients were documented and they or their legal representatives gave their consent. None of the subjects included in the study had been vaccinated for rotavirus. The stool samples were collected in sterile, screw cap containers and transferred to laboratory immediately under proper cold chain conditions.

Processing of stool specimens

10% stool suspension was prepared by dissolving 1 gm or 1 ml of stool in 10 ml of phosphate buffer saline (PBS) in a tube. The contents of the tube were vortexed for 1 min and then centrifuged at 10,000 rpm for 10 min to remove the coarse debris. The supernatant was then transferred to a fresh tube and stored at 4°C for short term and -80°C for long term.

Enzyme linked immunosorbent assay (ELISA)

Detection of rotavirus in the stool samples was done using commercially available enzyme linked immunosorbant assay kit (Premier Rotaclone, Meridian Biosciences Inc., Cincinnati, OH, USA) according to manufacturer's instructions.

RNA isolation with TRIzol method

Viral RNA was isolated from stool suspensions using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to previously published protocol with slight modifications. The RNA pellet was resuspended in RNase-free water and stored at -70°C until further use.

Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was prepared by reverse transcription of isolated RNA using Verso cDNA synthesis kit (ThermoFisher scientific, Waltham, Massachusetts, USA).

Polymerase chain reaction

Detection of rotavirus, norovirus and enterovirus

Three diarrhea causing RNA viruses, group A rotavirus, norovirus (GI & GII) and enterovirus were detected using PCR based method. The primers used for detection of various viruses were VP6(F) & VP6(R) for group A rotavirus; Mon432 & Mon434 for norovirus GI; Mon431 & Mon433 for norovirus GII; and F1 & R1 for enterovirus [40].

The thermo cycler conditions employed for the detection of aforementioned viruses are shown below:

Virus	T_{ID}		T_C		T_A		T_E		T_{FE}		No. of cycles
	Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (min)	
Rotavirus	95	5	95	30	48	30	68	30	68	10	35
Norovirus GI & GII	95	5	95	30	50	30	68	30	68	10	35
Enterovirus	95	5	95	30	48	30	68	45	68	10	35

T_{ID} = Initial Denaturation
T_C = Cyclic Denaturation
T_A = Annealing Temperature
T_E = Cyclic Extension
T_{FE} = Final Extension
min = Minutes
sec = Seconds

Rotavirus genotyping

The cDNA samples positive for VP6 based PCR were carried forward for genotyping by semi-nested PCR. The G and P typing was done using previously published primers recommended by World Health Organization with slight modifications [41, 42].

First round

For G genotyping, in the first round PCR, a 881 bp region of VP7 gene was amplified using VP7-F and VP7-R primers whereas for P genotyping, a region of 876 bp of VP4 gene was amplified using con2 and con3 primers in the first round PCR. The thermocycling conditions for the first round PCR were:

T_{ID}		T_C		T_A		T_E		T_{FE}		**No. of cycles**
Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (min)	
95	5	95	30	46	30	72	80	72	10	35

Second round PCR for G-genotyping

The first round product was subjected to the second round PCR using VP7-R as the reverse primer and a mixture of forward primers specific for G1, G2, G3, G4, G8, G9 and G10 genotypes. The thermocycling conditions for second round PCR for G-genotyping are following:

T_{ID}		T_C		T_A		T_E		T_{FE}		**No. of cycles**
Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (min)	
95	5	95	30	42	60	68	60	68	10	35

G12-specific second round PCR

The second round PCR for the detection of G12 genotype was carried out separately [43]. In this PCR the VP7-F was used as the forward primer whereas G12 specific primer was used in reverse orientation. The thermocycling conditions employed were:

T_{ID}		T_C		T_A		T_E		T_{FE}		**No. of cycles**
Temp. (^{0}C)	Time (min)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (sec)	Temp. (^{0}C)	Time (min)	
95	5	95	30	48	30	68	40	68	10	35

Second round PCR for P-genotyping

For P-genotyping of rotavirus, the first round products of VP4 specific PCR were subjected to the second round PCR where con3 was used as the forward primer and a mixture of P[4], P[6], P[8], P[9], P[10] and P[11] specific primers were used as reverse primers. The thermocycling conditions employed for second round PCR for P-genotyping were similar to the second round PCR for G-genotyping.

Sequencing and Phylogenetic analysis

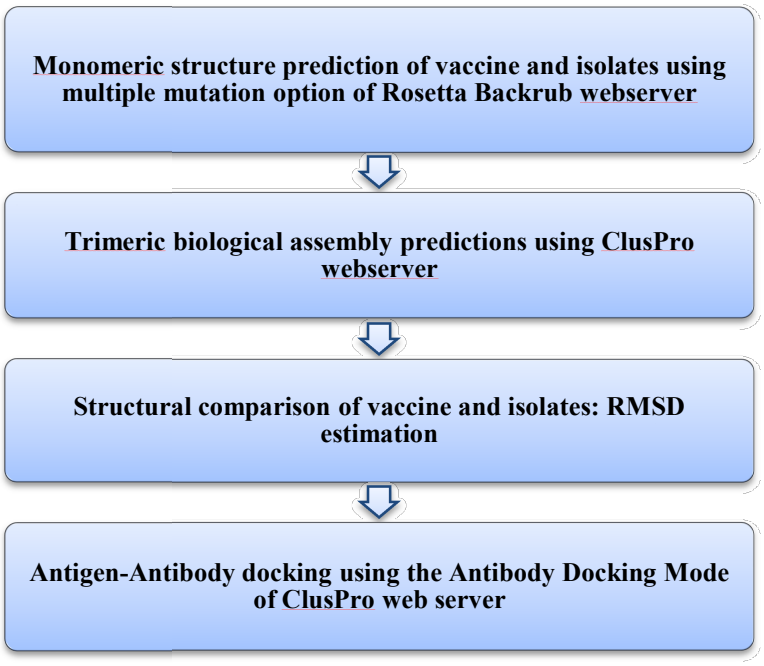
The amplified 881 bp RT-PCR products were sequenced (accession number KP938516, KP938517, KP938518, KP938519 and KM880064), aligned and analyzed with the corresponding VP7 sequences of rotavirus G1 strains available in GenBank using ClustalW. The phylogenetic analysis was carried out with MEGA software, version 6.0 (Tamura et al., 2013). The phylogenetic tree was constructed using the neighbor-joining method. The evolutionary distances were computed using the Kimura 2-parameter method. The statistical significance of the different phylogenetic groupings was estimated by bootstrap analysis with 1,000 pseudoreplicates.

Protein modeling and comparative analysis

The three dimensional protein structure of VP7 monomer was predicted using multiple point mutation feature of RosettaBackrub server. Suitable template was found by carrying out the similarity search in Protein Data Bank using blastp. For template, the crystal structure of VP7 protein was downloaded from Protein Data Bank by entering searching for '3FMG'. The RosettaBackrub server yielded 10 structures with different scores. The structure with least score was carried forward for oligomerization. The monomeric structures were oligomerized into trimeric form using ClusPro web server. The trimeric structures were validated using RAMPAGE server and the Ramachandran plot was generated of the proteins using SAVES server. The structures of the isolates and vaccines strains were compared by superimposing them in UCSF Chimera-Molecular Modeling System which gave the deviations by calculating Root Mean Square Deviation (RMSD) values.

Antibody docking analysis

The trimeric form of the VP7 protein of the isolates and vaccines were docked with the crystal structure of the anti-VP7 antibody available in association with the VP7 protein (3FMG) in the Protein Data Bank. The docking study was performed in the antibody mode of ClusPro server.


Monomeric structure prediction of vaccine and isolates using multiple mutation option of Rosetta Backrub webserver
Trimeric biological assembly predictions using ClusPro webserver
Structural comparison of vaccine and isolates: RMSD estimation
Antigen-Antibody docking using the Antibody Docking Mode of ClusPro web server

Work plan for *in silico* analysis

Statistical analysis

The prevalence of different viruses was estimated by the proportion of positive samples. Bivariate analysis of differences in clinical characteristics of rotavirus positive and rotavirus negative patients was done using Chi-square test. Difference in rotavirus infection in male and female patients was analyzed by Fisher's exact test. P values <0.05 were considered statistically significant.

Results and discussion

Incidences of diarrheagenic viruses in the sample population

In the first phase of study, a total of 287 stool samples were screened for group A rotavirus, norovirus (GI & GII) and enterovirus. In this phase, rotavirus emerged as the predominant virus isolated in almost 79 samples whereas norovirus and enterovirus were detected in very small fractions. Therefore, the further study (including screening and molecular characterization) was performed on rotaviruses only.

In the first phase, 99 (34.5%) out of 287 patients were positive for the viral pathogens (Table 1). Out of these, 97 (33.8%) patients were positive for single virus whereas mixed infection was found in 2 (0.6%) cases. Among single infections, rotavirus was the most prevalent viruses with 27.5% (79/287) positivity among all cases. Enterovirus was the second most predominant viruses (4.9%, 14/287) followed by norovirus GII (1.0%). Norovirus GI was the least detected virus with presence in only one (0.3%) sample. Two patients showing mixed infection had a coinfection of rotavirus and enterovirus in their stool samples.

Table 1: Frequency of diarrheagenic viruses in the sample population

Age Group	Rotavirus	Enterovirus	Norovirus GI	Norovirus GII	Coinfection (RV+EV)
<5 years (N=111)	55 (49.5%)	6 (5.4%)	1 (0.9%)	3 (2.7%)	–
>5 years (N=176)	24 (13.6%)	8 (4.5%)	0 (0.0%)	0 (0.0%)	2 (1.1%)
Total (N=287)	79 (27.5%)	14 (4.9%)	1 (0.3%)	3 (1.0%)	2 (0.7%)

Rotavirus prevalence: ELISA vs PCR

A total of 607 samples (including 287 from first phase) were investigated for the presence of rotavirus. 25.2% (153/607) were positive for rotavirus in ELISA whereas PCR-based method revealed positivity in 28.3% (172/607) samples. Considering PCR as a reference, the sensitivity of ELISA came out to be 84.5% and the specificity was found to be 98.6%. The positive predictive value and negative predictive value of 96.1% and 94.1% were obtained for the ELISA test. Among the children (≤5 years) samples, the rotavirus positivity was 49.0% (121/247) whereas 16.0% (8/50) samples from older children (6-17 years) had rotavirus in them. The frequency of rotavirus in adult patients was found out to be 13.9% (43/310). This prevalence of rotavirus is comparatively higher than the recent studies from different parts of the country where rotavirus positivity was found to be varying generally from 26%-40% [44-46]. However, there are studies from some parts of the country which reported the rotavirus prevalence of as high as 71% in Calicut [47], 53% in Kolkata [48] and 50% in Trichy [45] .

Distribution of diarrheagenic viruses in different age groups

(a) First phase

Rotavirus was the most commonly identified viruses in all age groups (Fig. 2). A major fraction of rotavirus infections were observed in the children up to 2 years of age. In this group, 50.0% (49/98) of the diarrheic patients were positive for rotavirus. The children belonging to the age group of 3-5 years showed 46.2% (6/13) positivity for rotavirus. Among the age groups of 6-17 years, 20.0% samples were positive for rotavirus. Rotavirus infections were lowest in adults and elderly belonging to the age groups of 18-65 and >65 years (15.4% and 5.0%, respectively). The distribution of enterovirus in different age groups was 5.1% in <2 years, 7.7% in 3-5 years, 15.0% in 6-17 years and 5.1% in 18-65 years of age. The elderly patients >65 years of age revealed no enterovirus infection. It is to be noted that among 6-17 and 18-65 age groups each had one case of rotavirus and enterovirus mixed infection. As far as norovirus infection is concerned, all the four cases were reported in children under the age of 2 years.

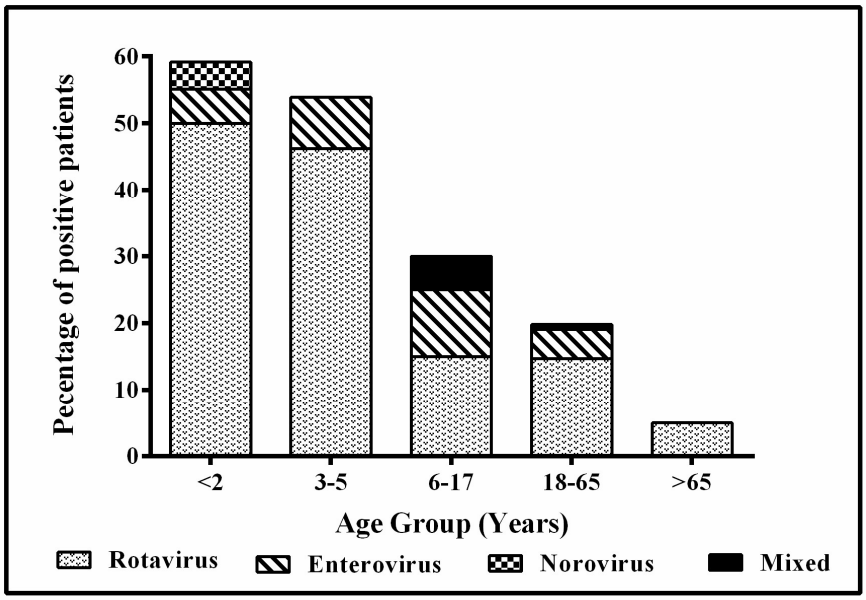


Fig. 2: Distribution of diarrheagenic viruses in the patients of different age groups

(b) Distribution of rotavirus in different age groups

The distribution of rotavirus in different age groups followed similar pattern as the first phase study (Fig. 3).

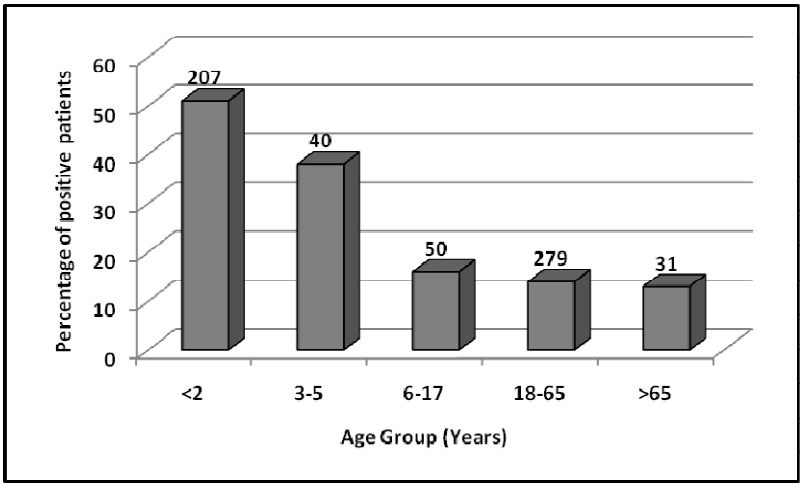


Fig. 3: Distribution of rotavirus in patients of different age groups. The figures in data labels represent number of samples screened

Studies have reported that the most rotavirus infections in children occurs before the age of 2 years [49-51] and in this study also the children <2 years of age demonstrated highest rate (51 %) of rotavirus infections among all the patients. This could be because of incompetence of the developing immune system of the children to set off the sufficient immune response which can combat the infecting pathogens. Studies have shown that the children between the age of 6-12 months have the declined level of maternal antibodies and are least protected against rotavirus infection [52, 53]. Also the children of this age group are in the process of crawling and walking stages where they could pick up the unhygienic materials in their mouth thus making them more exposed to the pathogens [54]. The relatively low infection rate of viral pathogens in older children may be due to the immunity developed because of previous exposures during the early stages of life [55].

Association of clinical features with rotavirus infection

As rotavirus was the predominant virus detected in both the children and adult patients, and it is extensively reported that rotavirus infections are commonly and significantly associated with the additional clinical symptoms along with diarrhea [56-58], clinical features of the rotavirus positive and rotavirus negative patients were compared to ascertain the association of rotavirus infection with the clinical features of vomiting, fever and dehydration (Table 2). In the children <5 years of age, 35.5% of rotavirus positive patients suffered from vomiting in contrast to the 23.0% of the non-rotavirus positive patients. This showed statistically significant association of vomiting with the rotavirus infection (P=0.0304). The statistically significant association of dehydration with rotavirus infection was also observed during the study (P=0.0240). Similarly, the rate of fever in the rotavirus positive cases were higher compared to rotavirus negative cases, but these differences showed no statistical significance. As a whole, these results are in concordance with the other studies which reported that rotavirus infected children suffer from more severe illness than children having non-rotavirus gastroenteritis [44, 54, 59-62]. On the contrary, in the age group >5 years, the rates of vomiting and fever in rotavirus infected individuals were lower in comparison with the patients having non-rotavirus diarrhea. In this age group, the rate of dehydration in rotavirus positive and rotavirus negative patients was almost similar. However, these results were not statistically significant. This leads to the conviction that

rotavirus infection results in more severe illness in children than adults and this might be an explanation for the higher rotavirus associated mortality in children than adults.

Table 2: Comparison of clinical characteristics of rotavirus positive and rotavirus negative patients

Age Group	Clinical Characteristics	Rotavirus Positive (%)	Rotavirus Negative (%)	P value
		N = 121	**N = 126**	
< 5 years	Vomiting	43 (35.5)	29 (23.0)	**0.030**
	Fever	31 (25.6)	26 (20.6)	0.353
	Dehydration	37 (30.6)	23 (18.3)	**0.024**
		N = 51	**N = 309**	
>5 years	Vomiting	7 (13.7)	63 (20.4)	0.265
	Fever	2 (3.9)	32 (10.4)	0.146
	Dehydration	10 (19.6)	59 (19.1)	0.931

Seasonal distribution of rotavirus infection

The infection rate of the gastroenteritis viruses in different seasons was studied. Among all the viruses under investigation, rotavirus infections were observed throughout the year with no particular seasonal peaks (Fig. 4). The highest prevalence of rotavirus was seen in the cold months of winter (november-february) where the overall (both children and adults) rotavirus positivity was observed in 38.5% samples. These results are in accordance with the other studies from northern India which shows maximum rotavirus infections and hospitalizations in the winter season [44, 46, 62-64]. But unlike those studies, rotavirus infection rate in this study was comparatively higher in the other seasons also. The season with second highest prevalence was summer (may-june) which experienced 29.4% rotavirus infectivity. The samples from autumn months (july-october) revealed least rotavirus positivity. The rotavirus infection in children was around 50% (± 5%) in all the seasons except autumn where only 39% of children were having rotavirus in their stool. This strange and different seasonality of rotavirus in Himachal Pradesh may be due to the geographical and climatic conditions of the region. Here the summers are not

very hot because of the high altitude and hilly terrain. Also the region experiences low relative humidity which is favorable for spread of rotavirus. The year round prevalence of rotavirus in the region warrants the implementation of continuous surveillance and preventive measures against the virus throughout the year and not only in particular seasons. Enterovirus and norovirus infections were found prominently in the winter and autumn seasons.

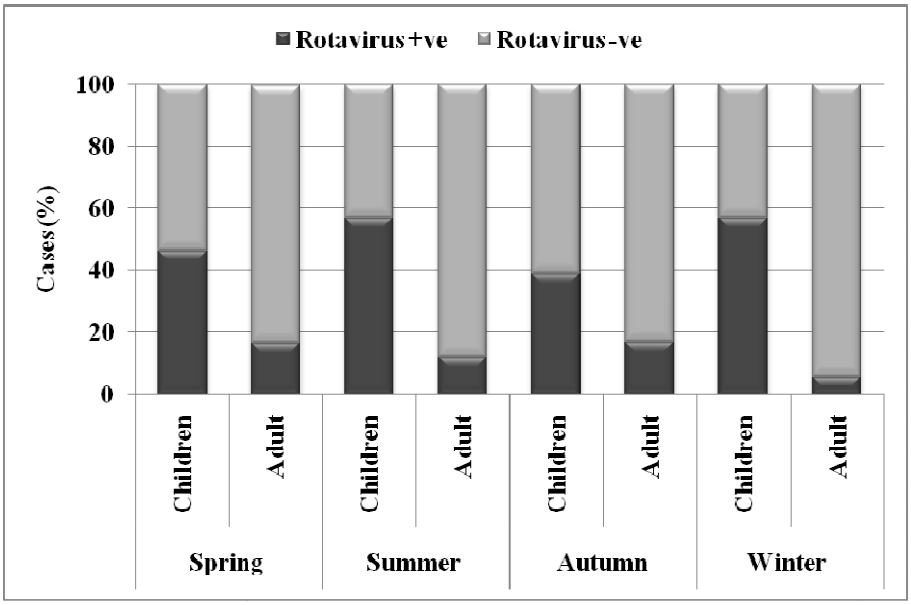


Fig. 4: Seasonal distribution of rotavirus infections

Rotavirus genotypes

Among G-types, G1 emerged as the predominant VP7 type with 66.0% (103/156) positivity (Table 3). The significance of high circulation of G1 lies in the fact that former studies have shown total absence of this genotype [39, 65]. G9 (11/156; 7.1%) was second most isolated genotype followed by G12 (6/156; 3.9%), G2 (1/156; 0.6%) and G10 (1/156; 0.6%). Mixed infection of G1/G9 and G9/G10 was observed in 3.2% (5/156) and 0.6% (1/156) samples, respectively. Among P-types identified, P[6] (104/156; 66.7%) was the most commonly identified type followed by P[8] (21/156; 13.5%). This high frequency of P[6] strains in Himachal Pradesh is not new to this region. Two previous reports on rotavirus diversity are available from this region and both the reports have documented significantly high rate (71.4%

and 97.3%) of P[6] circulation in the region [39, 65]. This suggests the continuous elevated prevalence of P[6] in the region from last two decades. Coinfection of P[6] and P[8] was seen in 2.6% (4/156) samples. As far as G and P combinations are concerned, interestingly, G1P[6] (76/121; 62.8%) was the most abundantly circulating rotavirus strain. The association of G1 with P[6] or any other genotype is considered unusual. According to our knowledge, this is the first time in India when we are witnessing such high infection rate attributed to G1P[6] rotavirus strains. The globally and nationally common G1P[8] (20/121; 16.5%) was second most frequently detected genotype. Among other strains, G9P[6] (9/121;7.4%), G12P[6] (6/121; 5.0%) and G2P[6] (1/121; 0.8%) were detected at significant frequency. Mixed genotypes accounted for 7.4% (9/121) of all G-P combinations.

Table 3: Characterization of rotavirus strains detected in the study

Genotype	P[6]	P[8]	P[6] & P[8]	P untyped	Total
G1	76	20	4	3	103
G2	1	0	0	0	1
G9	9	0	0	2	11
G10	0	0	0	1	1
G12	6	0	0	0	6
G1 & G9	4	1	0	0	5
G9 & G10	0	0	0	1	1
G untyped	8	0	0	20	28
Total	104	21	4	27	156

Distribution of genotypes in different age groups

The frequency pattern of circulating genotypes in different age groups was similar to the overall trend. G1P[6] remained the leading genotype in all age groups (Fig. 5). This particular genotype was identified in 56.7% (51/90) and 73.9% (17/23) of all genotyped samples in children and adults, respectively. In the 6-17 years group, all the samples (8/8; 100.0%) showed the presence of G1P[6] rotavirus strains. G1P[8] and G9P[6] were observed in both children and adult samples in notable fractions whereas G12P[6] and G2P[6] were detected only in the children samples. Mixed infections were evenly distributed in children and adult patients where each group had 8%-9% mixed infection cases.

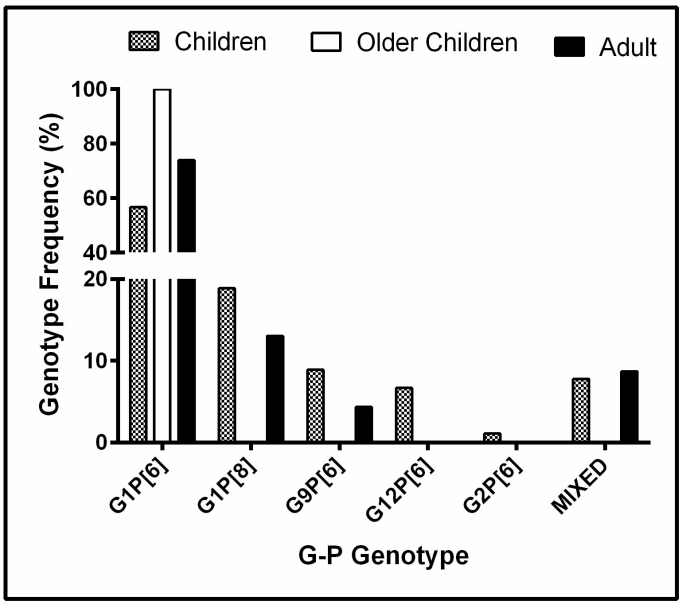


Fig. 5: Distribution of genotypes in different age groups

Phylogenetic and sequence analysis of the VP7 sequence of G1 rotavirus

Phylogenetic analysis of VP7 gene sequences (accession numbers KP938516, KP938517, KP938518, KP938519 and KM880064) showed that the G1 strains isolated from Himachal Pradesh clustered into G1-Lineage 1 (Fig. 6). But within G1-Lineage 1, these strains do not cluster with any of the reported five (A-E) sublineages and lack signature sequence of any of these. These results suggest that the isolated strains may belong to a novel sublineage other than the previously reported sublineages. Moreover, none of the isolates clustered with Rotarix (A41CB052A) and RotaTeq (W-179-9) vaccine strains which belong to the G1-Lineage 2 and G1-lineage 3, respectively. This may have an effect on vaccine efficacy. There are studies which show that intragenotypic nucleotide differences have a correlation with the antigenic differences. For instance, a study by Matthijnssens et al. reported that a rotavirus vaccinated child got infected by rotavirus G1 strain belonging to different lineage than the vaccine strain [66]. In another study Hoshino et al., proposed G9-lineage 1 rotavirus strains as more suitable vaccine candidate than strains belonging to lineage 2 or 3 [67].

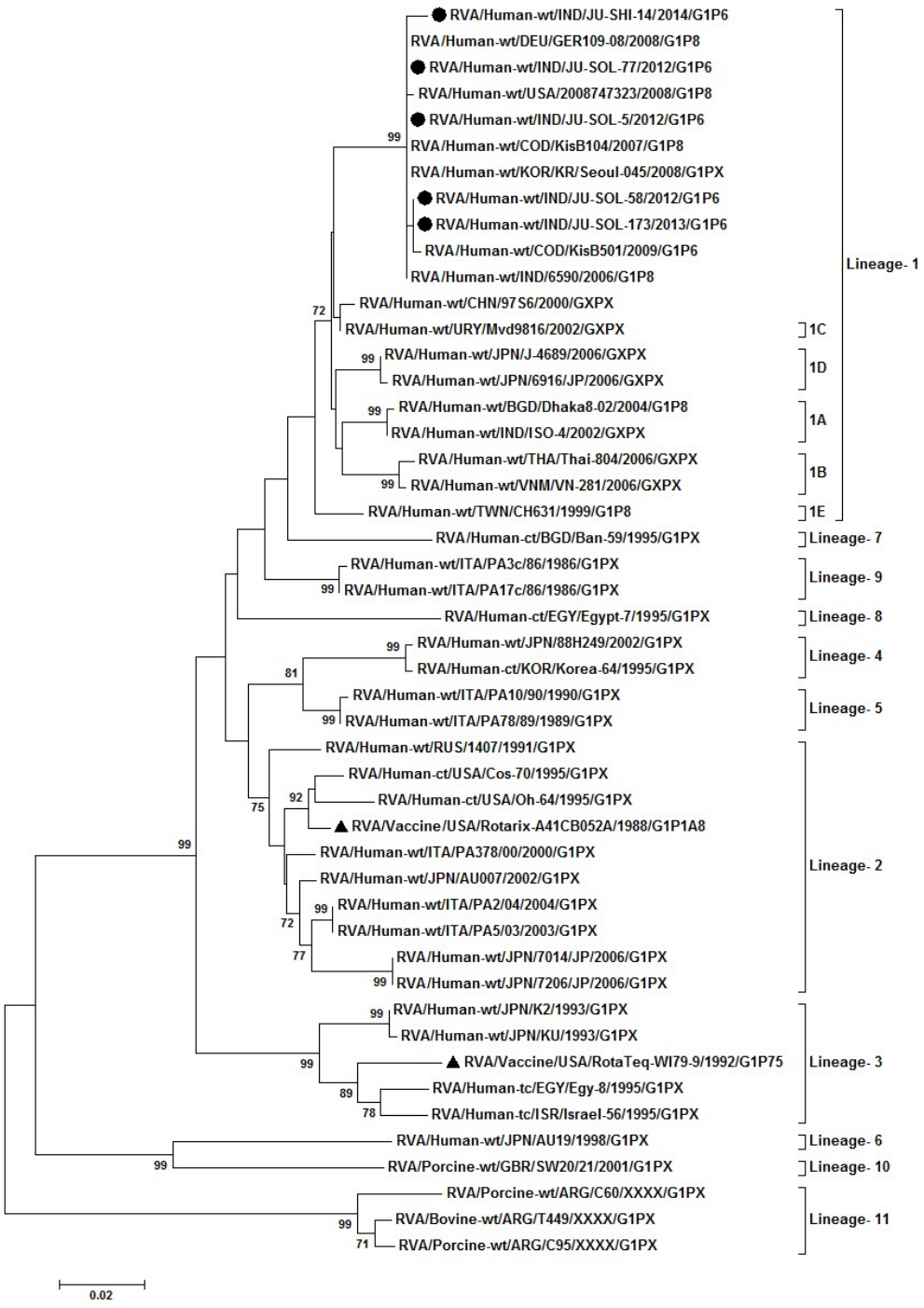


Fig. 6: Phylogenetic tree of VP7 gene sequences of rotavirus G1 strains. The phylogenetic tree was constructed using neighbour-joining method. The values at the branch nodes represent the bootstrap values. The Himachal isolates are indicated with black dots whereas vaccine sequences are indicated with black triangles. The accession numbers of the sequences used in the tree are mentioned in the text.

To assess the molecular basis of rotavirus diversity, the nucleotide and amino acid sequences of VP7 protein were compared. The isolates JU-SHI-14, JU-SOL-5, JU-SOL-58, JU-SOL-77, JU-SOL-173 were 99% identical to each other at nucleotide level. All the five isolates showed 96.1% to 97.6% and 96.4% to 98.8% similarity with the other lineage 1 strains on the nucleotide and amino acid levels, respectively. Within lineage 1, all the five isolates revealed the maximum identity (97.4%-97.8%) with the Uruguay strain, Mvd9816 at nucleotide level. However, on the amino acid level, the isolated strains shared maximum similarity with three strains, ISO-4 (98.2%-98.8%), VN-281 (98.2%-98.8%) and Mvd9816 (98.2%-98.8%), from India, Vietnam and Uruguay, respectively.

As reported by Phan et al. [68], there is a unique identification code for defining the lineages. It comprises of fourteen amino acids at positions 29, 34, 35, 37, 42, 43, 50, 55, 57, 65, 68, 72, 74 and 75 that define a signature sequence. The alignment of translated amino acid sequences of our strains revealed the signature code specific for lineage 1 (Table 4). Within lineage 1, the amino acid substitutions at positions 72 and 74 defines sublineage 1A, 16 defines 1B, 268 defines 1C and 91 defines 1D. Sublineage 1E has no sublineage specific mutations [68]. Interestingly, none of the isolates absolutely have the mutations specific for any of the sublineages within lineage 1. All the isolated strains showed a substitution of amino acid G with E at position 74 which is a characteristic feature for sublineage 1A. But there is no sublineage 1A specific amino acid substitution at position 72. This differentiates the isolates from the sublineage 1A specific strains. The strain JU-SHI-14 and JU-SOL-173 bears an additional substitution at position 91 and 268, respectively where amino acid T is substituted by A and I is replaced by V. These mutations support the fact that the Himachal isolates are diverse from the strains belonging to the five sublineages within lineage 1 and belong to a distinct sublineage.

Table 4: Lineage and sublineage specific amino acid substitutions in VP7 protein

Sublineages under Lineage 1	Strain	Amino Acid positions and substitutions																			
		16 I	29 I	34 I	35 Y	37 F	42 V	43 A	50 A	55 L	57 L	65 T	68 A	72 Q	74 G	75 I	91 T	211 N	212 V	213 D	268 I
1A	Dhaka 8-02	•	•	•	•	•	•	•	•	•	I	•	S	*R*	*E*	V	•	•	•	•	•
1B	Thai-804	*Q*	•	•	•	•	•	•	•	•	I	•	S	•	•	V	•	•	•	•	•
1C	Mvd9816	•	•	•	•	•	•	•	•	•	I	•	S	•	•	V	•	•	•	•	*V*
1D	6916	•	•	•	•	•	•	•	•	•	I	•	S	•	•	V	*N*	•	•	•	•
1E	CH631	•	•	•	•	•	•	•	•	•	I	•	S	•	•	V	•	•	•	•	•
	JU-SHI-14	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	*A*	•	•	•	-
	JU-SOL-5	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	•	•	•	•	-
	JU-SOL-58	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	•	-	-	-	-
	JU-SOL-77	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	•	•	•	•	-
	JU-SOL-173	•	•	•	•	•	•	•	•	•	I	•	S	•	*E*	V	•	•	•	•	*V*

Amino acids in *italics* represent sublineage specific mutations

Comparison of VP7 sequences of isolated G1 strains with the strains from other parts of India

The strains isolated in this study were compared with the other strains from different parts of India (Kolkata, Manipur, Pune and Haryana) on the basis of amino acid sequence of VP7 gene (Fig. 7). Most of the amino acid variations lie in the antigenic regions (Preferably, A and C). In the region A, a Himachal isolate, JU-SHI-14 showed the amino acid transition of T91A, whereas all the isolates from this study showed the substitution of amino acid E instead of D at position 100. Region C had three positions where Himachal isolates differ from the strains from other parts of the country. Strain JU-SOL-5 showed maximum amino acid differences at three positions (T210K, T217I and A219D). Two other strains, JU-SOL-77 and JU-SOL-173 were also having T217I transition. The isolates did not have any amino acid variations in the antigenic region B. Outside the antigenic regions, Himachal strains showed variations at two positions, 41 and 179 where F is replaced by S and G is replaced by E, respectively.

Comparison of VP7 sequences of isolated G1 strains and vaccine strains

The comparison of isolated strains with Rotarix vaccine strain showed 92.8% - 93.3% and 93.9% - 94.5% similarity at nucleotide and amino acid level, respectively. Similarly, the isolated strains revealed 87% - 87.5% nucleotide and 91.9% - 92.6% amino acid similarity with the RotaTeq strain. These results demonstrate that the isolated strains are closer to the Rotarix strain on the nucleotide and amino acid levels than RotaTeq vaccine strains.

Comparison of VP7 epitopes of isolated G1 strains and vaccine strains

The amino acid differences in the antigenic epitope region between circulating strains and vaccine strains could have a substantial effect on the vaccine efficacy [69]. Therefore, the antigenic regions of the isolated G1 strains and vaccine strains were compared to ascertain the variability between these. The isolated strains showed five amino acid differences in the epitopic region as compared with Rotarix strain, whereas RotaTeq showed a difference of seven amino acids (Table 5). Majority of the amino acid changes were found in epitope region 7-1a and it had amino acid changes at five positions. In this region, there was a transition between T→A, N→S, D (RotaTeq) →E, D→E and S→N at positions 91, 94, 97, 100 and 123, respectively. The region 7-2 has S (RotaTeq) →N and M→T/I amino acid change at positions 147 and 217, respectively. The significance of these mutations lies in the

fact that they are the neutralization escape mutation sites except amino acid at position 123 [34]. These amino acid differences could influence antigenicity of the strains and might give

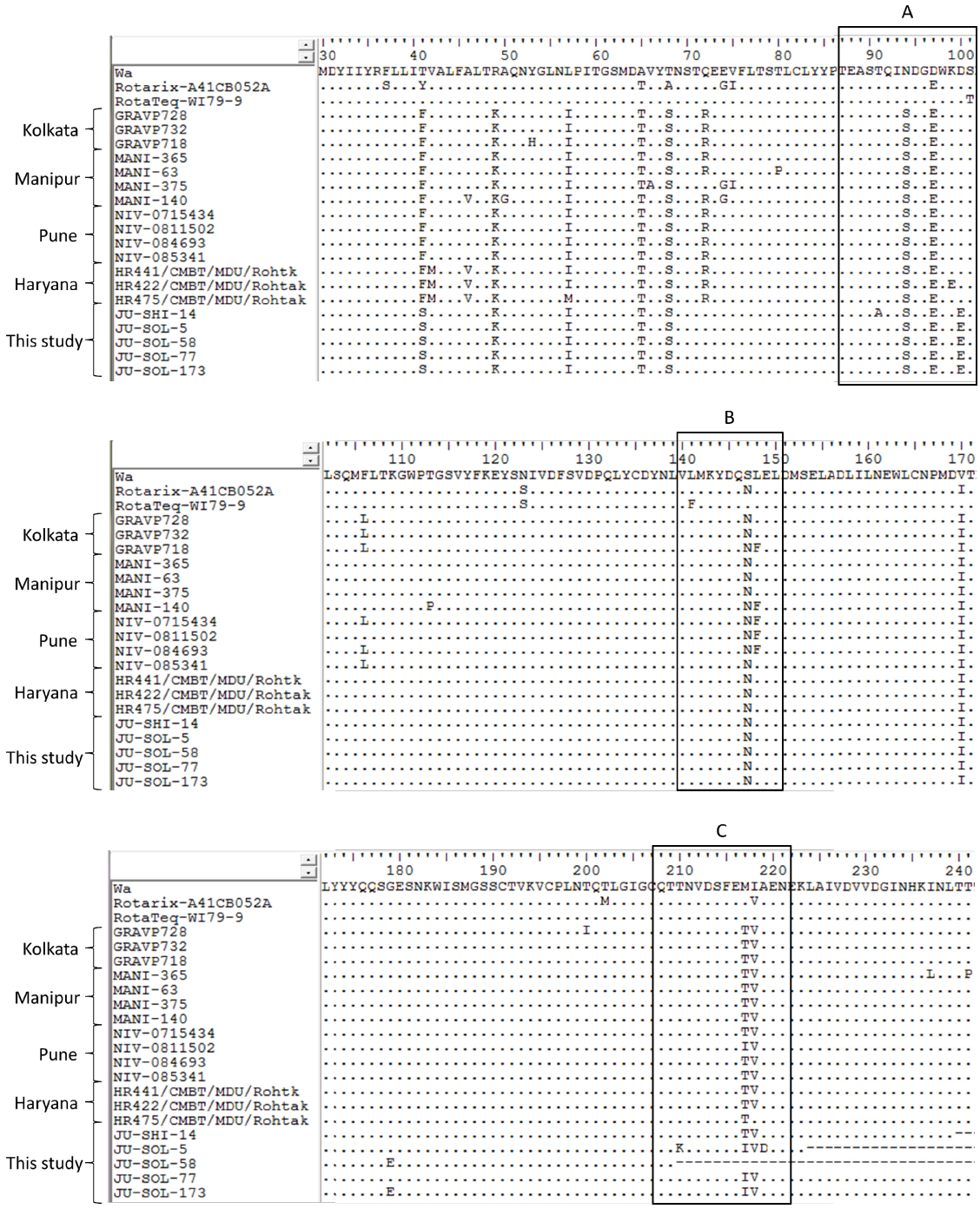


Fig. 7: Alignment of amino acid sequences of VP7 protein of G1-Himachal isolates and strains from other parts of the country. Dots represent the conserved amino acid residues. The antigenic regions are indicated by black boxes.

Table 5: Alignment of antigenic amino acids in VP7 protein of Himachal isolates and vaccine strains

Rotavirus Strain	Epitope 7-1a														Epitope 7-1b						Epitope 7-2								
	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
Rotarix-G1	**T**	**T**	**N**	**G**	**E**	**W**	**K**	**D**	**Q**	**S**	**V**	**V**	**D**	**K**	**Q**	**N**	**V**	**D**	**N**	**T**	**K**	**D**	**Q**	**N**	**L**	**S**	**M**	**N**	**G**
RotaTeq-G1	**T**	**T**	**N**	**G**	**D**	**W**	**K**	**D**	**Q**	**S**	**V**	**V**	**D**	**K**	**Q**	**N**	**V**	**D**	**N**	**T**	**K**	**D**	**Q**	**S**	**L**	**S**	**M**	**N**	**G**
JU-SHI-14	T	A	S	G	E	W	K	E	Q	N	V	V	D	-	Q	N	V	D	N	-	K	D	Q	N	L	S	T	N	-
JU-SOL-5	T	T	S	G	E	W	K	E	Q	N	V	V	D	-	Q	N	V	D	-	-	K	D	Q	N	L	S	I	N	-
JU-SOL-58	T	T	S	G	E	W	K	E	Q	N	V	V	D	-	Q	-	-	-	-	-	K	D	Q	N	L	S	-	-	-
JU-SOL-77	T	T	S	G	E	W	K	E	Q	N	V	V	D	-	Q	N	V	D	N	T	K	D	Q	N	L	S	I	N	-
JU-SOL-173	T	T	S	G	E	W	K	E	Q	N	V	V	D	-	Q	N	V	D	N	T	K	D	Q	N	L	S	I	N	-

Yellow color represents amino acid differences between Rotarix and RotaTeq. Magenta represents residues that differ from both Rotarix and RotaTeq. Cyan represents amino acid residues that differ from RotaTeq.

rise to neutralization antibody escape mutants [69, 70]. As far as region 7-1b is concerned, it was found to be fully conserved between circulating strains and vaccine strains. Mapping of the amino acid variations in the antigenic regions of the isolates and vaccines on the trimeric structure of VP7 protein reveals that the differences were located inconsistently on the exposed surfaces of the protein in the epitope region (Fig. 8).

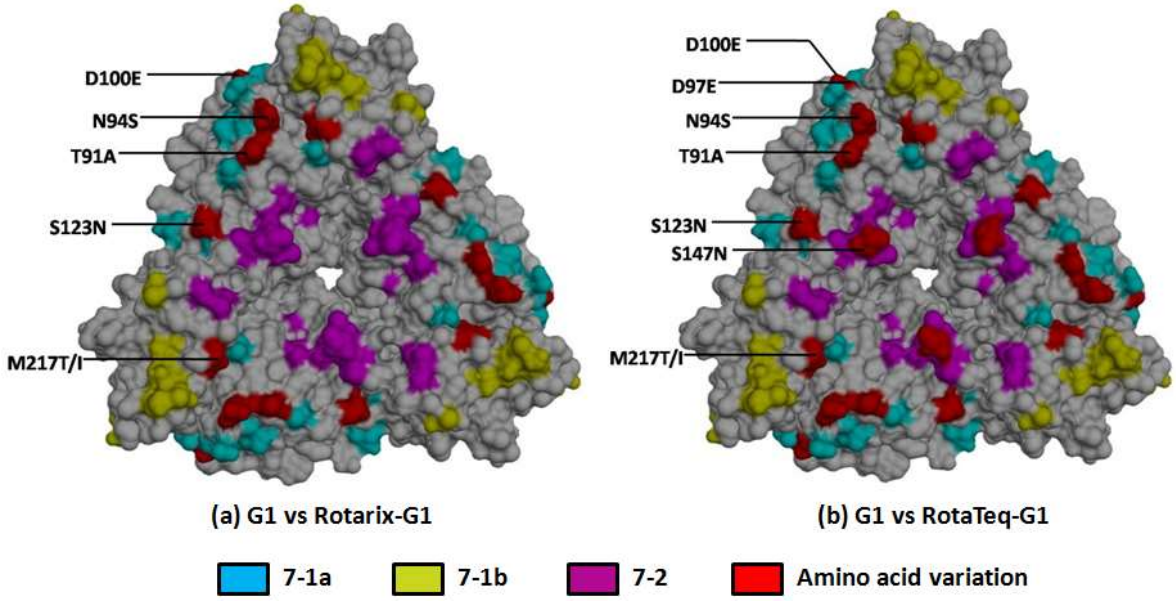


Fig. 8: Structural representation of VP7 trimer proteins. Different regions of antigenic epitopes are shown in cyan (7-1a), yellow (7-1b) and magenta (7-2) colors. The amino acid differences between Himachal strains and vaccine strains are indicated with red color.

Protein modeling and comparative analysis

The 3D structures of vaccine and rotavirus strains were predicted using Rosetta Backrub Server. The structure of 3FMG was made to undergo mutations at the specific position of Rotarix-G1, RotaTeq-G1, Rotavac-G9, JU-SHI-14, JU-SOL-5 and JU-SOL-58. The changes in JU-SOL-77 and JU-SOL-173 were similar to JU-SOL-5, so its results could be implemented on JU-SOL-77 and JU-SOL-173 respectively. The mutations were carried out using multiple point mutation feature of Rosetta Backrub Server. It predicted 10 structures with different weighted score. The

structure with minimum balanced score was carried forward for trimerization as it had maximum stability.

For trimerization, the pdb files of monomer units were subjected to cluspro server which yielded 15 structures with different weighted scores. The structure with least weighted score was selected for each protein and carried forward for comparative and docking analysis. All the trimers showed good stereochemical qualities and have 98.3%-99.0% residues in the allowed and favored regions of the Ramachandran plot (Data not shown).

To study the effect of amino acid variation on the structure of the proteins, the isolates were compared with the vaccines by superimposing them. The RMSD is the deviation of the position of atoms between the superimposed structures. The RMSD value of ≤ 2 Å signifies no significant differences between the superimposed structures. In the study, on comparing the isolates individually with Rotarix, RotaTeq and Rotavac, no significant difference in the protein structure was observed (Fig. 9).

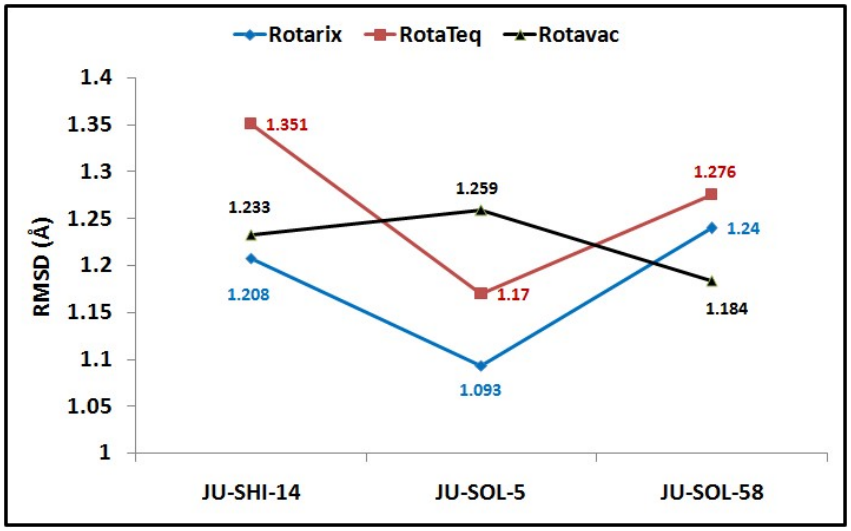


Fig. 9: Comparison of vaccine strains with rotavirus strains using RMSD

The possible explanation for this can be the chemical characteristics of the substituting amino acids. On observing the chemical characteristics of the amino acids under study, we can see that all the amino acids which are varying between the isolates and vaccines are having similar chemical characteristics except in isolate JU-SHI-14.

Antibody docking analysis

The protein structures of the isolates and vaccines were subjected to antibody docking, where the effect of amino acid variations on antibody binding was studied. The weighted scores obtained for the antigen antibody complex of the vaccines and isolates showed no considerable differences (Fig. 10).

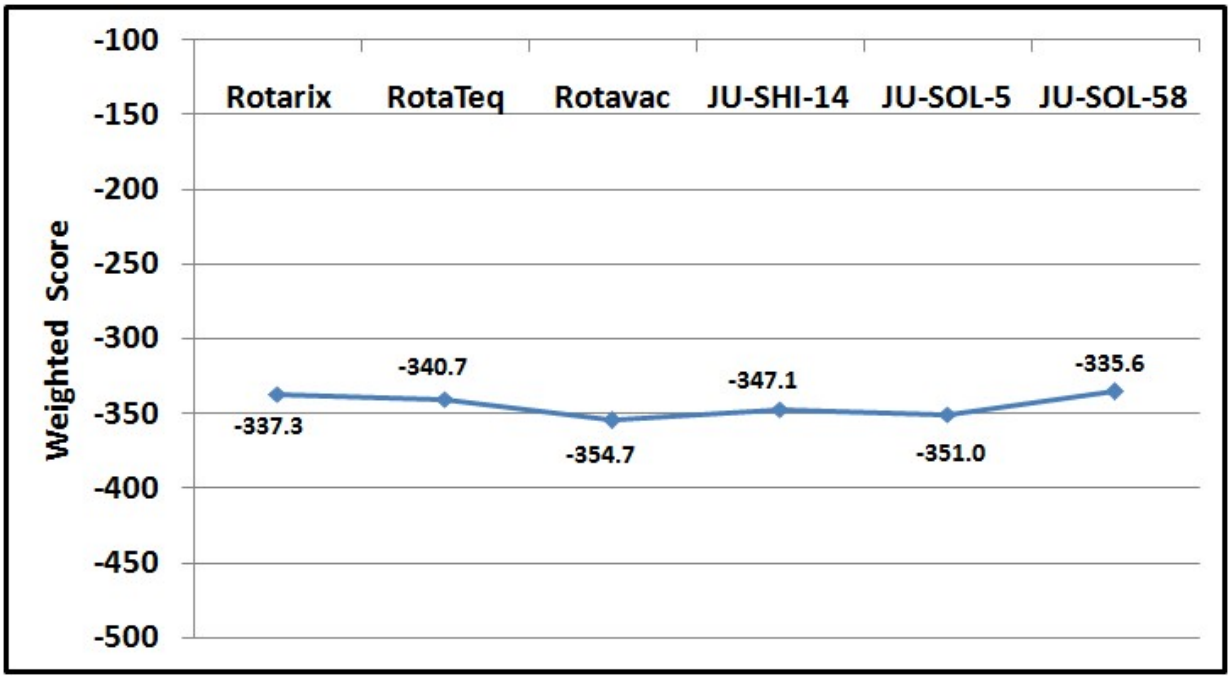


Fig. 10: Scoring function in the form of weighted score obtained after antibody docking analysis

This reflects that in spite of amino acid differences, the isolates behave in a similar fashion as vaccine, as far as their interaction with antibody in concerned. Thus, it can be concluded on the basis of this basic in silico study that the antibody binding capability of the isolates retains in the similar manner as the vaccines and thus it can be hypothesized that on the administration of these vaccines in the children of Himachal Pradesh there will be no negative effect on vaccine efficacy. Although this is merely an assumption which can be made, but actual inference can only be obtained after the availability of post vaccination data. However, before the availability of post vaccination data, serological analysis by wet lab experiments can generate more reliable

information on this aspect. This study is limited by the fact that the only anti-VP7 antibody structure available in PDB were raised against rotavirus G3 strain and used for the present *in silico* docking study. The more reliable results can only be obtained once the anti-G1 antibody structure is available and then used for docking studies against G1 strains.

Conclusion

This study not only throws light on the relative prevalence of the important diarrheagenic viruses but also furnish preliminary data which would be helpful in formulating the preventive strategies against them. In particular, the high infection rate of rotavirus in children and its year round prevalence warrants immediate attention in order to reduce the morbidity and suspected mortality due to this pathogen. The predominance of unusual rotavirus genotype, G1P[6] in the region under investigation is the crucial finding of the study and is indicative of bizarre diversity of rotavirus in the region. Moreover, the identification of potential antibody escape mutants during the study draws attention.

References

[1] World Health Organization. http://www.who.int/gho/mortality_burden_disease/causes_death/en/. 2015.

[2] Eckardt AJ, Baumgart DC. Viral gastroenteritis in adults. Recent Pat Antiinfect Drug Discov. 2011;6:54-63.

[3] Wilhelmi I, Roman E, Sanchez-Fauquier A. Viruses causing gastroenteritis. Clin Microbiol Infect. 2003;9:247-62.

[4] Rovida F, Campanini G, Piralla A, Adzasehoun KM, Sarasini A, Baldanti F. Molecular detection of gastrointestinal viral infections in hospitalized patients. Diagn Microbiol Infect Dis. 2013;77:231-5.

[5] Thongprachum A, Takanashi S, Kalesaran AF, Okitsu S, Mizuguchi M, Hayakawa S, et al. Four-year study of viruses that cause diarrhea in Japanese pediatric outpatients. J Med Virol. 2015;87:1141-8.

[6] Banajeh SM, Abu-Asba BA. The epidemiology of all-cause and rotavirus acute gastroenteritis and the characteristics of rotavirus circulating strains before and after rotavirus vaccine introduction in Yemen: analysis of hospital-based surveillance data. BMC Infect Dis. 2015;15:015-1165.

[7] Tate JE, Burton AH, Boschi-Pinto C, Parashar UD, Agocs M, Serhan F, et al. Global, regional, and national estimates of rotavirus mortality in children< 5 years of age, 2000–2013. Clin Infect Dis. 2016;62:S96-S105.

[8] World Health Organization. http://www.wpro.who.int/mediacentre/factsheets/fs_20120229/en/. 2012.

[9] John J, Sarkar R, Muliyil J, Bhandari N, Bhan MK, Kang G. Rotavirus gastroenteritis in India, 2011-2013: revised estimates of disease burden and potential impact of vaccines. Vaccine. 2014;11:004.

[10] Anderson EJ, Weber SG. Rotavirus infection in adults. Lancet Infect Dis. 2004;4:91-9.

[11] Nakajima H, Nakagomi T, Kamisawa T, Sakaki N, Muramoto K, Mikami T, et al. Winter seasonality and rotavirus diarrhoea in adults. Lancet. 2001;357:1950.

[12] Lanata CF, Fischer-Walker CL, Olascoaga AC, Torres CX, Aryee MJ, Black RE. Global causes of diarrheal disease mortality in children <5 years of age: a systematic review. PLoS One. 2013;8.

[13] Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinje J, Parashar UD. Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerg Infect Dis. 2008;14:1224-31.

[14] Kroneman A, Harris J, Vennema H, Duizer E, van Duynhoven Y, Gray J, et al. Data quality of 5 years of central norovirus outbreak reporting in the European Network for food-borne viruses. J Public Health. 2008;30:82-90.

[15] Zheng DP, Widdowson MA, Glass RI, Vinje J. Molecular epidemiology of genogroup II-genotype 4 noroviruses in the United States between 1994 and 2006. J Clin Microbiol. 2010;48:168-77.

[16] Pons-Salort M, Parker EP, Grassly NC. The epidemiology of non-polio enteroviruses: recent advances and outstanding questions. Curr Opin Infect Dis. 2015;22:22.

[17] Harada S, Okada M, Yahiro S, Nishimura K, Matsuo S, Miyasaka J, et al. Surveillance of pathogens in outpatients with gastroenteritis and characterization of sapovirus strains between 2002 and 2007 in Kumamoto Prefecture, Japan. J Med Virol. 2009;81:1117-27.

[18] Patil PR, Chitambar SD, Gopalkrishna V. Molecular surveillance of non-polio enterovirus infections in patients with acute gastroenteritis in Western India: 2004–2009. J Med Virol. 2015;87:154-61.

[19] Rao DC, Ananda Babu M, Raghavendra A, Dhananjaya D, Kumar S, Maiya PP. Non-polio enteroviruses and their association with acute diarrhea in children in India. Infect Genet Evol. 2013;17:153-61.

[20] Scarcella C, Carasi S, Cadoria F, Macchi L, Pavan A, Salamana M, et al. An outbreak of viral gastroenteritis linked to municipal water supply, Lombardy, Italy, June 2009. Euro surveill. 2009;14:506-13.
[21] Matthijnssens J, Otto PH, Ciarlet M, Desselberger U, Van Ranst M, Johne R. VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. Arch Virol. 2012;157:1177-82.
[22] Matthijnssens J, Ciarlet M, McDonald SM, Attoui H, Bányai K, Brister JR, et al. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). Arch Virol. 2011;156:1397-413.
[23] Rotavirus Classification Working Group (RCWG). Newly assigned genotypes. (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg). 2016.
[24] da Silva Soares L, de Fátima dos Santos Guerra S, do Socorro Lima de Oliveira A, da Silva dos Santos F, de Fátima Costa de Menezes E, Mascarenhas JdAP, et al. Diversity of rotavirus strains circulating in Northern Brazil after introduction of a rotavirus vaccine: High prevalence of G3P [6] genotype. J Med Virol. 2014;86:1065-72.
[25] Esona MD, Armah GE, Steele AD. Rotavirus VP4 and VP7 genotypes circulating in Cameroon: identification of unusual types. J Infect Dis. 2010;202:S205-S11.
[26] Gurgel RQ, Alvarez ADJ, Rodrigues A, Ribeiro RR, Dolabella SS, Da Mota NL, et al. Incidence of rotavirus and circulating genotypes in Northeast Brazil during 7 years of national rotavirus vaccination. PloS One. 2014;9:e110217.
[27] Piekarska A, Kacerka A, Majda-Stanisławska E, Jóźwiak B, Sidorkiewicz M. Predominance of genotype P [9] G3 in rotavirus gastroenteritis in Polish children. Arch Med Sci: AMS. 2015;11:577-83.
[28] World Health Organization (WHO). Global Rotavirus Surveillance and Information Bulletin. 2015;10:1–4.
[29] Jain S, Vashistt J, Changotra H. Rotaviruses: Is their surveillance needed? Vaccine. 2014;32:3367-78.
[30] Trinh QD, Nguyen TA, Phan TG, Khamrin P, Yan H, Hoang PL, et al. Sequence analysis of the VP7 gene of human rotavirus G1 isolated in Japan, China, Thailand, and Vietnam in the context of changing distribution of rotavirus G-types. J Med Virol. 2007;79:1009-16.
[31] Dyall-Smith ML, Lazdins I, Tregear GW, Holmes IH. Location of the major antigenic sites involved in rotavirus serotype-specific neutralization. Proc Natl Acad Sci U S A. 1986;83:3465-8.
[32] Kobayashi N, Taniguchi K, Urasawa T, Urasawa S. Analysis of the neutralization epitopes on human rotavirus VP7 recognized by monotype-specific monoclonal antibodies. J Gen Virol. 1991;72:1855-61.
[33] Nishikawa K, Hoshino Y, Taniguchi K, Green KY, Greenberg HB, Kapikian AZ, et al. Rotavirus VP7 neutralization epitopes of serotype 3 strains. Virology. 1989;171:503-15.
[34] Aoki ST, Settembre EC, Trask SD, Greenberg HB, Harrison SC, Dormitzer PR. Structure of rotavirus outer-layer protein VP7 bound with a neutralizing Fab. Science. 2009;324:1444-7.
[35] Wang CM, Chen SC, Chen KT. Current status of rotavirus vaccines. World J Pediatr. 2015;11:300-8.
[36] Ciarlet M, Schodel F. Development of a rotavirus vaccine: clinical safety, immunogenicity, and efficacy of the pentavalent rotavirus vaccine, RotaTeq. Vaccine. 2009;30:107.
[37] Kulkarni R, Arora R, Chitambar SD. Sequence analysis of VP7 and VP4 genes of G1P[8] rotaviruses circulating among diarrhoeic children in Pune, India: a comparison with Rotarix and RotaTeq vaccine strains. Vaccine. 2014;11:080.
[38] Ramachandran M, Das BK, Vij A, Kumar R, Bhambal S, Kesari N, et al. Unusual diversity of human rotavirus G and P genotypes in India. J Clin Microbiol. 1996;34:436-9.

[39] Jain V, Das BK, Bhan MK, Glass RI, Gentsch JR. Great diversity of group A rotavirus strains and high prevalence of mixed rotavirus infections in India. J Clin Microbiol. 2001;39:3524-9.
[40] Jain S, Thakur N, Grover N, Vashistt J, Changotra H. Prevalence of rotavirus, norovirus and enterovirus in diarrheal diseases in Himachal Pradesh, India. Virusdisease. 2016;27:77-83.
[41] Gentsch J, Glass R, Woods P, Gouvea V, Gorziglia M, Flores J, et al. Identification of group A rotavirus gene 4 types by polymerase chain reaction. J Clin Microbiol. 1992;30:1365-73.
[42] Iturriza-Gomara M, Kang G, Gray J. Rotavirus genotyping: keeping up with an evolving population of human rotaviruses. J Clin Virol. 2004;31:259-65.
[43] Kheyami AM, Nakagomi T, Nakagomi O, Dove W, Hart CA, Cunliffe NA. Molecular epidemiology of rotavirus diarrhea among children in Saudi Arabia: first detection of G9 and G12 strains. J Clin Microbiol. 2008;46:1185-91.
[44] Saluja T, Sharma S, Gupta M, Kundu R, Kar S, Dutta A, et al. A multicenter prospective hospital-based surveillance to estimate the burden of rotavirus gastroenteritis in children less than five years of age in India. Vaccine. 2014;32:A13-A9.
[45] Babji S, Arumugam R, Sarvanabhavan A, Moses PD, Simon A, Aggarwal I, et al. Multi-center surveillance of rotavirus diarrhea in hospitalized children< 5 years of age in India, 2009–2012. Vaccine. 2014;32:A10-A2.
[46] Kang G, Desai R, Arora R, Chitamabar S, Naik TN, Krishnan T, et al. Diversity of circulating rotavirus strains in children hospitalized with diarrhea in India, 2005–2009. Vaccine. 2013;31:2879-83.
[47] Broor S, Ghosh D, Mathur P. Molecular epidemiology of rotaviruses in India. Indian J Med Res. 2003;118:59.
[48] Mullick S, Mandal P, Nayak MK, Ghosh S, De P, Rajendran K, et al. Hospital based surveillance and genetic characterization of rotavirus strains in children (< 5 years) with acute gastroenteritis in Kolkata, India, revealed resurgence of G9 and G2 genotypes during 2011–2013. Vaccine. 2014;32:A20-A8.
[49] Albano F, Bruzzese E, Bella A, Cascio A, Titone L, Arista S, et al. Rotavirus and not age determines gastroenteritis severity in children: a hospital-based study. Eur J Pediatr. 2007;166:241-7.
[50] Salinas B, Gonzalez G, Gonzalez R, Escalona M, Materan M, Schael IP. Epidemiologic and clinical characteristics of rotavirus disease during five years of surveillance in Venezuela. Pediatr Infect Dis J. 2004;23:S161-7.
[51] Velazquez FR, Matson DO, Calva JJ, Guerrero L, Morrow AL, Carter-Campbell S, et al. Rotavirus infections in infants as protection against subsequent infections. N Engl J Med. 1996;335:1022-8.
[52] Zheng B, Ma G, Tam J, Lo S, Ng M, Lam B, et al. The effects of maternal antibodies on neonatal rotavirus infection. Pediatr Infect Dis J. 1991;10:865.
[53] Mata L, Simhon A, Urrutia J, Kronmal RA, Fernandez R, Garcia B. Epidemiology of rotaviruses in a cohort of 45 Guatamalan Mayan Indian children observed from birth to the age of three years. J Infect Dis. 1983;148:452-61.
[54] Paul A, Gladstone BP, Mukhopadhya I, Kang G. Rotavirus infections in a community based cohort in Vellore, India. Vaccine. 2014;32:A49-A54.
[55] Moyo SJ, Gro N, Kirsti V, Matee MI, Kitundu J, Maselle SY, et al. Prevalence of enteropathogenic viruses and molecular characterization of group A rotavirus among children with diarrhea in Dar es Salaam Tanzania. BMC Public Health. 2007;7:1.
[56] Lieberman JM. Rotavirus and other viral causes of gastroenteritis. Pediatr Ann. 1994;23:529-32.

[57] Sai L, Sun J, Shao L, Chen S, Liu H, Ma L. Epidemiology and clinical features of rotavirus and norovirus infection among children in Ji'nan, China. Virol J. 2013;10:10-302.
[58] Saravanan P, Ananthan S, Ananthasubramanian M. Rotavirus infection among infants and young children in Chennai, South India. Indian J Med Microbiol. 2004;22:212-21.
[59] Chitambar SD, Ranshing SS, Pradhan GN, Kalrao VR, Dhongde RK, Bavdekar AR. Changing trends in circulating rotavirus strains in Pune, western India in 2009–2012: Emergence of a rare G9P [4] rotavirus strain. Vaccine. 2014;32:A29-A32.
[60] Namjoshi GS, Mitra M, Lalwani SK, Sachdeva A, Balasubramanian S, Babji S, et al. Rotavirus gastroenteritis among children less than 5 years of age in private outpatient setting in urban India. Vaccine. 2014;32:A36-A44.
[61] Alkali BR, Daneji A, Magaji A, Bilbis L. Clinical symptoms of human rotavirus infection observed in children in Sokoto, Nigeria. Adv Virol. 2015.
[62] Kang G, Arora R, Chitambar SD, Deshpande J, Gupte MD, Kulkarni M, et al. Multicenter, hospital-based surveillance of rotavirus disease and strains among indian children aged <5 years. J Infect Dis. 2009;1:605031.
[63] Jagai JS, Sarkar R, Castronovo D, Kattula D, McEntee J, Ward H, et al. Seasonality of rotavirus in South Asia: a meta-analysis approach assessing associations with temperature, precipitation, and vegetation index. PloS One. 2012;7:e38168.
[64] Mathew A, Rao PS, Sowmyanarayanan TV, Kang G. Severity of rotavirus gastroenteritis in an Indian population: report from a 3 year surveillance study. Vaccine. 2014;32:A45-A8.
[65] Ramachandran M, Das BK, Vij A, Kumar R, Bhambal SS, Kesari N, et al. Unusual diversity of human rotavirus G and P genotypes in India. J Clin Microbiol. 1996;34:436-9.
[66] Matthijnssens J, Bilcke J, Ciarlet M, Martella V, Banyai K, Rahman M, et al. Rotavirus disease and vaccination: impact on genotype diversity. Future Microbiol. 2009;4:1303-16.
[67] Hoshino Y, Jones RW, Ross J, Honma S, Santos N, Gentsch JR, et al. Rotavirus serotype G9 strains belonging to VP7 gene phylogenetic sequence lineage 1 may be more suitable for serotype G9 vaccine candidates than those belonging to lineage 2 or 3. J Virol. 2004;78:7795-802.
[68] Phan TG, Khamrin P, Quang TD, Dey SK, Takanashi S, Okitsu S, et al. Detection and genetic characterization of group A rotavirus strains circulating among children with acute gastroenteritis in Japan. J Virol. 2007;81:4645-53.
[69] O'Ryan ML, Matson DO, Estes MK, Pickering LK. Anti-rotavirus G type-specific and isotype-specific antibodies in children with natural rotavirus infections. Journal of Infectious Diseases. 1994;169:504-11.
[70] Green K, Kapikian AZ. Identification of VP7 epitopes associated with protection against human rotavirus illness or shedding in volunteers. J Virol. 1992;66:548-53.

Publications

- **Jain S,** Vashistt J, Gupta K, Kumar A, Changotra H. Molecular analysis of VP7 gene of rotavirus G1 strains isolated from Himachal Pradesh, North India. *Current Microbiology* 2016; DOI: 10.1007/s00284-016-1129-2. (Impact factor: 1.5)

- **Jain S,** Thakur N, Grover N, Vashistt J, Changotra H. Prevalence of rotavirus, norovirus and enterovirus in diarrheal diseases in Himachal Pradesh, India. *VirusDisease* 2016;27(1):77-83. (Impact factor: 0.4)

- **Jain S,** Vashistt J, Changotra H. Rotaviruses: Is their surveillance needed?. *Vaccine* 2014;32:3367-78. (Impact factor: 3.4)

- **Jain S,** Thakur N, Vashistt J, Grover N, Krishnan T, Changotra H. Predominance of unusual rotavirus G1P[6] strain in North India: an evidence from hospitalized children and adult diarrheal patients. *Infection, Genetics and Evolution*. (Under review after revision) (Impact factor: 2.8)

- Nutan, **Jain S**, Shilpa, Tomar A, Changotra H, Vashistt J. Computational Tools; Indispensable Armamentarium of Medical Biotechnology. *Indian Journal of Science and Technology* 2016;9(32):1-5.

Conferences

- **Jain S,** Vashistt J, Changotra H (2015). Predominance of Rotavirus G1P[6] strain in Himachal Pradesh, India. *Proceedings of the 5th Asian Vaccine Conference.* Held at Hanoi, Vietnam on 12-14 June 2015. (Travel grant of Rs. 71,600/- from ICMR, India)

- **Jain S,** Singh TR, Vashistt J, Changotra H (2015). Molecular analysis of G1 rotavirus strains isolated from Himachal Pradesh. *Proceedings of the National Conference on Emerging Trends in Host-Microbe Interactions.* Held at Jalandhar, India on 17-18 April 2015.

- **Jain S**, Kumar N, Thakur N, Vashistt J, Changotra H (2014). Phylogenetic analysis to study the evolution of Rotavirus G1P[6] strain isolated from Himachal Pradesh. *Proceedings of the International Conference on Stem Cell Research, Cancer Biology, Biomedical Sciences, Bioinformatics and Applied Biotechnology.* Held at New Delhi, India on 1 -2 November 2014.

- Nutan, **Jain S**, Changotra H, Shrivastava R, Vashistt J (2015). Metagenomic Studies in Relation to Diarrheal Diseases. *Proceedings of the International Congress on Friedreichs Ataxia and DNA Structure in Health & Disease*. Held at New Delhi, India on 11-13 April , 2015.

- Nutan, **Jain S,** Grover N, Kumar Y, Changotra H, Vashistt J (2014). Identification, characterization of prevalent diarrheagenic bacterial pathogens from regions of Himachal Pradesh. *Proceedings of the International Conference on Life science Biotechnology & Bioinformatics* Held at Noida, India on 29-30 August 2014.

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